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Metabolism of Hydroxypyruvate in a Mutant of Barley Lacking NADH-Dependent Hydroxypyruvate Reductase, an Important Photorespiratory Enzyme Activity[1]

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ABSTRACT

A mutant of barley (*Hordeum vulgare* L.), LaPr 88/29, deficient in NADH-dependent hydroxypyruvate reductase (HPR) activity has been isolated. The activities of both NADH (5%) and NADPH-dependent (19%) HPR were severely reduced in this mutant compared to the wild type. Although lacking an enzyme in the main carbon pathway of photorespiration, this mutant was capable of CO_2 fixation rates equivalent to 75% of that of the wild type, in normal atmospheres and 50% O_2. There also appeared to be little disruption to the photorespiratory metabolism as ammonia release, CO_2 efflux and $^{14}CO_2$ release from L-[U-^{14}C]serine feeding were similar in both mutant and wild-type leaves. When leaves of LaPr 88/29 were fed either [^{14}C]serine or $^{14}CO_2$, the accumulation of radioactivity was in serine and not in hydroxypyruvate, although the mutant was still able to metabolize over 25% of the supplied [^{14}C]serine into sucrose. After 3 hours in air the soluble amino acid pool was almost totally dominated by serine and glycine. LaPr 88/29 has also been used to show that NADH-glyoxylate reductase and NADH-HPR are probably not catalyzed by the same enzyme in barley and that over 80% of the NADPH-dependent HPR activity is due to the NADH-dependent enzyme. We also suggest that the alternative NADPH activity can metabolise a proportion, but not all, of the hydroxypyruvate produced during photorespiration and may thus form a useful backup to the NADH-dependent enzyme under conditions of maximal photorespiration.

Photorespiration is the apparently wasteful production of CO_2 in the light by C_3 plants and appears to be an unavoidable consequence of the O_2 sensitivity of ribulose bisphosphate carboxylase (10, 17). Carboxylation of ribulose bisphosphate produces two molecules of phosphoglycerate while oxygenation produces only one molecule of phosphoglycerate and one of phosphoglycolate. Phosphoglycolate is metabolized further by the interlocking photorespiratory carbon and nitrogen cycles traversing three compartments of the cell (7, 11). Within this pathway, hydroxypyruvate is an important intermediate produced in the peroxisome by the action of SGAT[3] and subsequently reduced to glycerate under the auspices of HPR.

At atmospheric concentrations of CO_2 greater than 0.2%, carbon is directed mainly through the carboxylase activity of ribulose bisphosphate carboxylase/oxygenase. This property was utilized by Somerville and Ogren (22) to identify and maintain conditional lethal mutants, with deficiencies in the photorespiratory pathway and consequently incapable of growth at ambient levels of CO_2 (0.034%).

Definitive studies of HPR activity in plants are hampered by the apparent multiplicity of enzymes showing this activity (12, 13). The central importance in photorespiration of the NADH-dependent peroxisomal enzyme HPR has been accepted for almost 20 years (10, 11, 29) and is an enzyme thought to be especially active in green leaves (25, 29). Recently, a novel HPR activity has been isolated and purified from spinach that can utilize NADPH in preference to NADH as a cofactor (12). This form of HPR has been shown to be localized in the cytosol (14). The existence of glyoxylate reductase activity is also partially confusing.

Although mutants deficient in some of the many steps of photorespiration have been isolated from populations of mutated seed of *Arabidopsis thaliana* (22), barley (3), pea (3), tobacco (19) and *Chlamydomonas* (26), this is the first report of a mutant of any species that lacks NADH-dependent HPR activity.

MATERIALS AND METHODS

Plant Material

The M_2 seeds of *Hordeum vulgare* L. (cv Klaxon) used for mutant screening were produced as described previously (20) in a glasshouse supplemented to a CO_2 concentration of 0.7% (v/v). Selection of air-sensitive plants was carried out in a glasshouse with atmospheric CO_2 concentrations, maintained at 20°C.

Preparation of Crude Enzyme Extract

The preparation of crude enzyme extracts for use in measurements of enzyme activity and with PAGE was carried out as described by Murray *et al.* (20).

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[3] Abbreviations: SGAT, serine: glyoxylate aminotransferase; GR, glyoxylate reductase; HPR, hydroxypyruvate reductase.

Assay of Hydroxypyruvate and Glyoxylate Reductase Activity

The assays for NADH and NADPH-HPR activities were based on those described by Stabenau (24) and Kleczkowski and Randall (12). The final concentrations in 1 mL of assay mixture were 5 mM hydroxypyruvate, 25 mM phosphate buffer (pH 6.3), 0.2 mM NADH (NADPH) and 50 μL crude enzyme extract. Reactions were initiated by the addition of hydroxypyruvate and the oxidation of NADH (NADPH) was monitored at 340 nm.

GR activity was determined using the same assay system by the substitution of 5 mM glyoxylate for hydroxypyruvate in the reaction mixture.

PAGE

Native PAGE and staining for NADH-HPR activity were carried out using 4.5% acrylamide slab gels (15, 27), other related reductases not being reversible are not detected by this method (12). Samples were loaded onto gels after mixing with 10% glycerol and bromophenol blue.

The activity stain was performed in the direction of glycerate oxidation and resulted in the appearance of reduced nitroblue tetrazolium as a purple precipitate. The activity stain was carried out for 30 min in the dark using the following assay mixture; 200 mM Tris (pH 8.9), 58 mM DL-glycerate, 0.33 mM nitroblue tetrazolium, 2.2 mM NAD, and 0.081 mM phenazine methosulfate. The reaction was terminated by the addition of 7.5% (v/v) acetic acid.

Measurement of CO_2 Assimilation

Rates of gas exchange were measured on detached leaves as described previously (2), using a photon fluence rate of 1700 μmol m^{-2} s^{-1} and a flow rate of 400 cm^3 min^{-1}.

L-[U-^{14}C]Serine Feeding, $^{14}CO_2$ Feeding, and $^{14}CO_2$ Efflux

L-[U-^{14}C]Serine feeding and measurement of $^{14}CO_2$ efflux were carried out as described elsewhere (20) using $^{14}CO_2$ in air (0.04%, 12 mCi/mmol) at a Pfr of 800 μmol m^{-2} s^{-1} at 20°C. $^{14}COOH_2$ was also fed to detached leaves using the same apparatus. Soluble products of photosynthesis and photorespiration were extracted (21) separated on two dimensional TLC and analysed by autoradiography and scintillation counting (30). All results are quoted on a percentage basis of the radioactivity taken up by the leaves.

Measurement of Amino Acid Content and Ammonia Content

Amino acids were extracted from leaf tissue and analysed as *o*-phthaldialdehyde derivatives by HPLC (20). Ammonia was measured after extraction in 0.1 M HCl, using phenol hypochlorite (18).

Other Procedures

The soluble protein content of the extracts was measured by the method of Bradford (4), against a BSA standard.

RESULTS

Isolation of LaPr 88/29

During a screen of air-sensitive barley plants one plant, LaPr 88/29, was selected that contained less than 5% of the wild-type NADH-dependent HPR activity (Table I).

Further investigation into the activities of HPR and GR in crude leaf extracts, using either NADH or NADPH as a source of reductant, showed that in LaPr 88/29 the activities of NADH-HPR and NADPH-HPR were drastically reduced compared to the wild type, whereas the activities of NADH-GR and NADPH-GR were only slightly altered (Table I).

The levels of some other photorespiratory enzyme activities (glutamine synthetase, SGAT, glutamate:glyoxylate aminotransferase, and glycerate kinase) were also investigated in leaves of LaPr 88/29 and were not different from the wild-type plant (data not shown).

Native PAGE

A single band of NADH-HPR activity was clearly visible when partially purified extracts of the wild type were subjected to native PAGE. The intensity of the stain for this activity was linearly related to the amount of protein applied to the gel (Fig. 1). There was no evidence of any NADH-HPR activity in LaPr 88/29, even at a protein loading of 1.0 mg per lane.

CO_2 Fixation Rate and CO_2 Efflux

Leaves of LaPr 88/29 exhibited the same rate of CO_2 fixation as wild-type leaves when placed in 1% O_2, 0.034%

Table I. *Activities of HPR and GR in Crude Leaf Extracts of Wild-Type Barley or LaPr 88/29 Using Either NADH or NADPH as Source of Reductant*

All values are the mean of three measurements and are quoted as nkat mg^{-1} protein.

Enzyme Activity	Wild-Type	LaPr 88/29
NADH HPR	2.34	0.12
NADH GR	0.51	0.40
NADPH HPR	1.39	0.27
NADPH GR	0.35	0.32

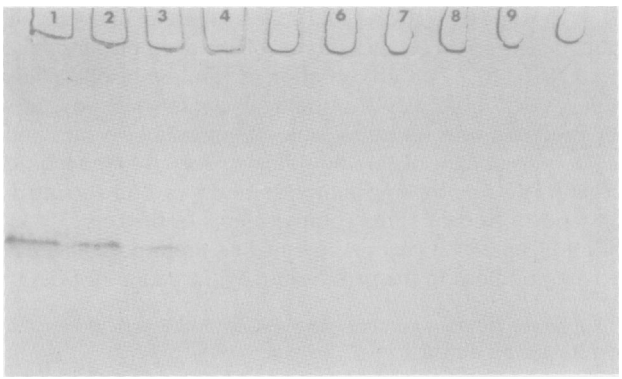

Figure 1. HPR activity stain on 4.5% acrylamide gels for wild-type barley (tracks 1–4; 150, 75, 50 and 25 μg protein) and LaPr 88/29 (tracks 6–9).

CO_2, but on transfer to 21% O_2, 0.034% CO_2, the rate of the mutant fell to 75% of the wild-type rate (Fig. 2). Transfer to 50% O_2, 0.034% CO_2 depressed the fixation rate even further in both the mutant and the wild type, with the mutant rate remaining at approximately 75% of the wild type.

The rates of CO_2 fixation measured in 21% O_2 and 0.034% CO_2 for both the wild-type and the mutant plants remained constant for at least 4 h. Longer times were not investigated although it was possible to grow the mutant in air at a Pfr of 200 μmol m^{-2} s^{-1} for up to 8 weeks with only minor leaf tip necrosis.

There was no difference between the wild-type and mutant plants in the rate of CO_2 efflux into CO_2-free air at varying levels of O_2 in the gas phase (data not shown).

L-[U-^{14}C]Serine Feeding and $^{14}CO_2$ Efflux

When L-[U-^{14}C]serine was fed to leaves of the wild type and LaPr 88/29 (Table II), over half the total radioactivity recovered in the mutant was found in serine (52.8%), compared with less than 20% in the wild type. The accumulation of ^{14}C in glycine by LaPr 88/29 was double that of the wild type

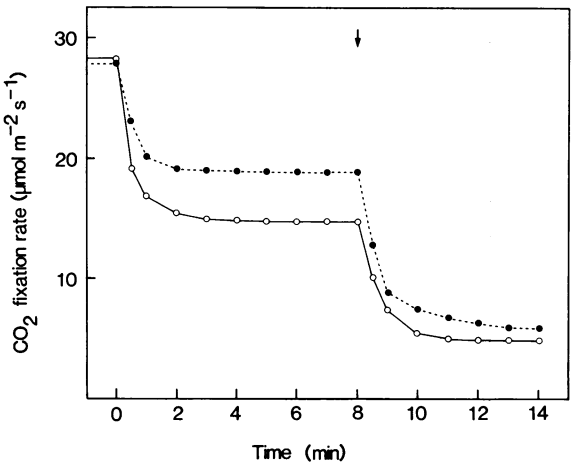

Figure 2. Rate of CO_2 fixation by detached leaves of wild-type barley (●----●) and LaPr 88/29 (○——○) after transfer to air at time zero and to 50% O_2 at the *arrow*.

Table II. *Distribution of ^{14}C from L-[U-^{14}C]labeled Serine in Leaves of Wild-Type and LaPr 88/29 Barley after CO_2 Fixation for 60 min in Air*

All values are expressed as a percentage of ^{14}C uptake.

Extract	Wild-Type	LaPr 88/29
Sugars	60.7	25.3
Sugar phosphates	0.9	1.0
Phosphoglycerate	1.3	0.8
Organic acids	4.9	4.6
Malate	1.0	n.d.
Glycolate	0.4	1.2
Glycine	2.8	5.4
Serine	19.6	52.8
Glycerate	1.0	2.0
CO_2 released	7.4	6.9

(Table II). Although there was a block in the carbon pathway of photorespiration in this mutant, some of the supplied L-[U-^{14}C]serine was metabolized into the sugar fraction (25%) compared with a value of 60.7% for the wild type.

The $^{14}CO_2$ efflux from leaves of the wild type and LaPr 88/29 supplied with L-[U-^{14}C]serine under photorespiratory conditions showed no differences (data not shown).

$^{14}CO_2$ Feeding

Feeding $^{14}CO_2$ to leaves of wild type and LaPr 88/29 after photosynthetic induction, gave an accumulation of almost four times as much ^{14}C in serine in the mutant compared to the wild type, whereas the amount of ^{14}C in sucrose was only reduced slightly (56 to 45%) by the mutation (Table III). When $^{14}CO_2$ was supplied to the plants during the first 5 min of photosynthetic induction (Table IV), twice as much serine accumulated in the mutant and more ^{14}C was found in the sugar fraction than in the wild type.

Amino Acid Accumulation

After transfer from a higher level of CO_2 (0.7%) to air, wild-type barley accumulated glutamate, glutamine, serine and glycine over the 3 h period studied (Fig. 3). However, when LaPr 88/29 was transferred from high CO_2 to air, the amino acid pattern was dominated after 3 h by serine (71%) and glycine (15%), the other amino acids only totalled 14% (Fig.

Table III. *Distribution of Radioactivity after $^{14}CO_2$ Fixation for 5 min in Air, by Leaves of Wild-Type and LaPr 88/29 Barley*

Before $^{14}CO_2$ was supplied in air, CO_2 fixation was fully induced by exposure to 1% O_2, 0.034% CO_2 for 60 min. Figures are expressed as a percentage of the total recovered counts.

Extract	Wild-Type	LaPr 88/29
Sugars	56.4	45.4
Sugar phosphates	3.9	3.9
Phosphoglycerate	6.4	3.5
Organic acids	14.0	9.5
Glycolate	0.7	1.6
Glycine	10.9	6.4
Serine	7.7	29.7

Table IV. *Distribution of Radioactivity after $^{14}CO_2$ Fixation for 5 min in Air by Leaves of Wild-Type and LaPr 88/29 Barley*

Before $^{14}CO_2$ was supplied the plants had been placed in the dark for at least 60 min. Figures are expressed as a percentage of the total recovered counts.

Extract	Wild-Type	LaPr 88/29
Sugars	15.7	24.7
Sugar phosphates	14.0	7.7
Phosphoglycerate	20.4	10.4
Organic acids	10.5	3.3
Glycolate	1.0	0
Glycine	9.6	8.8
Serine	17.8	34.6
Glycerate	5.7	4.7
Phospho*enol*pyruvate	1.0	2.0
Other amino acids	4.3	3.8

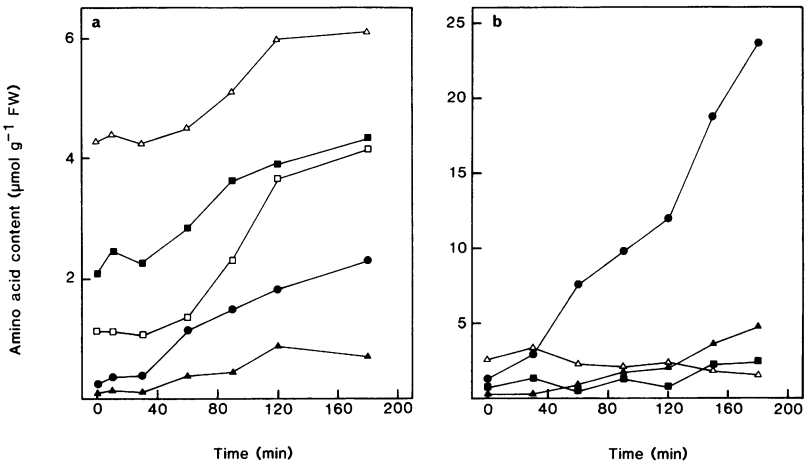

Figure 3. Change in leaf amino acid content with time in wild-type barley (a) and LaPr 88/29 (b) following transfer to air for serine (●), glycine (▲), alanine (■), glutamate (△), and glutamine (□).

3). Glutamine was not detected in the mutant even after 24 h in air (data not shown).

Ammonia Accumulation

Methionine sulfoximine supplied to leaves of the wild-type and mutant plant showed that there was no difference in the rate of ammonia evolution between the two lines (data not shown).

DISCUSSION

An air-sensitive barley plant, LaPr 88/29, was identified as having less than 5% of wild-type NADH-dependent HPR activity (Table I). Hydroxypyruvate is produced in the peroxisome from serine, and in LaPr 88/29 there was an accumulation of serine, in fact 86% of the total extractable amino acids were found in serine and glycine after only 3 h exposure to air. When plants were fed $^{14}CO_2$ in air (Table III) after photosynthetic induction, LaPr 88/29 accumulated almost four times as much ^{14}C in serine as the wild type with the amount of ^{14}C found in sucrose in LaPr 88/29 being 85% of the wild type, showing that the mutation had caused only a slight reduction. Presumably, ^{14}C accumulates in serine because any surplus hydroxypyruvate not reduced would be transaminated back to serine by available amino donors.

When HPR activity was assayed using a mixture of wild-type and mutant extracts, the rate of NADH oxidation was the same as for the wild type alone (data not shown). Thus, it was not the presence of an inhibitor in the mutant that caused the reduction of HPR activity detected in LaPr 88/29.

Activity staining of nondenaturing gels revealed only one band of activity in purified extracts of cucumber (8, 27). Evidence from Figure 1 and Kleczkowski and Randall (12) clearly shows only one band of NADH-HPR activity, as revealed by activity staining or immunoblotting in wild-type barley or spinach leaves. Similar staining of gels on which extracts of LaPr 88/29 were run failed to provide any evidence of NADH-HPR activity.

The residual NADH-HPR activity reported in Table I presumably reflects an alternative activity of the novel NADPH-dependent enzyme (12). Further support for this is provided by a comparison of the NADH and NADPH-dependent activities in Table I. It had previously been reported (12) that the NADPH-HPR activity would use NADH at 50% of the NADPH-dependent rate and Table I shows that the ratio of NADH to NADPH-dependent activities in LaPr 88/29 was approximately 50%.

It has often been assumed that NADH-GR and NADH-HPR are identical (13, 29). Evidence from Table I, where NADH-dependent GR activity in the mutant plant was reduced by only 22% compared to the wild type, would strongly suggest that the majority of NADH-GR activity is not catalyzed by the same enzyme as NADH-HPR activity in crude extracts of barley.

From Table I it is also possible to evaluate the contribution of the peroxisomal NADH-dependent enzyme to the total NADPH-dependent activity. If it is assumed that in LaPr 88/29 there was only one mutation that affected the NADH-dependent form of HPR, then the difference between the wild-type and the mutant rates would reflect the proportion of NADPH activity that was due to the NADH-dependent enzyme. It thus appeared that 81% of the total NADPH-dependent activity in wild-type barley plants, was due to the NADH-dependent enzyme. This figure confirms the earlier speculation based on measurements of NADH and NADPH-dependent HPR in spinach (12), that between 75 and 85% of the total NADPH-dependent activity was due to the NADH-dependent enzyme.

Although LaPr 88/29 lacked an enzyme in the main photorespiratory carbon pathway, under photorespiratory conditions the rate of CO_2 fixation was still 75% of the wild-type rate. Barley mutants lacking catalase, which is not in the main photorespiratory pathway (9), showed similar photosynthetic characteristics to LaPr 88/29, but other mutants which had lesions in the main carbon pathway, *e.g.* phosphoglycolate phosphatase, glycine decarboxylase and SGAT (3), have invariably shown fixation rates of less than 30% of the wild type within a few minutes of transfer to photorespiratory conditions. Apparently, LaPr 88/29 was capable of metabolizing hydroxypyruvate by another route. As expected, when the O_2 concentration flowing over the leaves was increased to 50% and thus forcing more carbon through photorespiration, the

CO_2 fixation rates of both wild-type and LaPr 88/29 fell. The mutant still, however, maintained a fixation rate of 75% of the wild type. The accumulation of ammonia, which is produced during photorespiration in equivalent amounts to CO_2 production, was also little different in the wild-type and mutant plants. Clearly the disruption of photorespiratory metabolism in LaPr 88/29 was not as great as might be expected with a mutation in a major photorespiratory enzyme. Surprisingly, when fed L-[U-^{14}C]serine (Table II), 25% of the detected ^{14}C was still recovered as sugars, equivalent to 41% of the amount recovered in sugars from the wild type. This demonstrated that the mutant was able to bypass the lesion in the peroxisome by another route.

One possible alternative pathway for the utilization of hydroxypyruvate is pyruvate decarboxylase, which in wheat germ (5), is also capable of decarboxylating hydroxypyruvate yielding CO_2 and glycolate. However, metabolism of hydroxypyruvate by such a mechanism seems unlikely in LaPr 88/29 even when the major enzyme for hydroxypyruvate metabolism is absent, since measurement of $^{14}CO_2$ release from [^{14}C] serine feeding and from CO_2 efflux at varying oxygen concentrations, showed no enhanced CO_2 efflux compared to the wild type under photorespiratory conditions.

When the novel NADPH-HPR from spinach was initially purified and characterised (12), it was only possible to speculate on its possible functions. Kleczkowski and Randall (12) suggested that like its NADH-dependent counterpart, the enzyme was responsible for recovery of carbon skeletons. The subsequent report that NADPH-HPR was located in the cytosol (14), and the previous knowledge that hydroxypyruvate was able to 'leak' out of the peroxisome (1, 16) enhanced this speculation.

LaPr 88/29 provides a means for unrestricted study of the NADPH-dependent enzyme. Results from several studies over a number of years (6, 23) have demonstrated that the rate of photorespiratory loss *in vivo* was approximately 40% of the net rate of CO_2 fixation. From the data in Figure 2 this would imply for LaPr 88/29 a maximal photorespiratory rate of 7 μmol m^{-2} s^{-1} (or 0.47 nmol s^{-1} mg^{-1}). Table I shows that the residual activity, measured under maximal conditions in LaPr 88/29 was 0.27 nmol s^{-1} mg^{-1} protein. This suggested that although the alternative pathway could probably reduce part of the photorespiratory flux of hydroxypyruvate, there was insufficient activity to deal with the whole flux under high rates of photorespiration.

It would appear, therefore, from the evidence collected using LaPr 88/29, the first reported mutant plant of any species lacking NADH-HPR activity, that these speculations may be correct and that the alternative NADPH activity may form a useful backup to the NADH-dependent enzyme, especially when photorespiration is operating at its full rate, *e.g.* under conditions of high light intensity (28).

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